BIOLOGICAL DETOXIFICATION OF FOODBORNE BACTERIAL EXOTOXINS USING SELECTED PROBIOTIC STRAINS

A

Synopsis

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1.0 INTRODUCTION

1.1 Foodborne diseases

One of the major global problems today is Food Security. Food and Agriculture Organization (FAO) states that *'Food security exists when all people, at all times, have physical, (social) and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life'* (Page *et al.*, 2002).Safety here implies food free of the entire range of hazards that can make food noxious to health.. Table 1.0 shows the different common hazards of each category. Food hazards can be broadly classified into Physical Hazards, Chemical Hazards & Biological Hazard.

Table 1 Types of Food Hazards

Physical Hazard	Glass, Metal, Insect parts, etc.
Chemical Hazard	Mycotoxins, Pesticide residues, Heavy metals etc.
Biological Hazard	Bacteria, Fungi, Virus, Protozoans, Helminthes etc.

(Source : UNLIVER FOOD SOLUTION, 2021)

1.2Types of Foodborne Diseases

According to the World Health Organization, foodborne diseases are defined as diseases of infectious or toxic nature, which are caused by the consumption of contaminated food or water. They are caused by infectious or toxic agents that enter the body through ingestion of food (Adams *et al*., 2003). Foodborne diseases are also caused by pathogens; these pathogenic organisms can be transmitted through contaminated foods (Vemula *et al*., 1989). Figure-1.0 depicts the foodborne diseases caused by microorganisms.

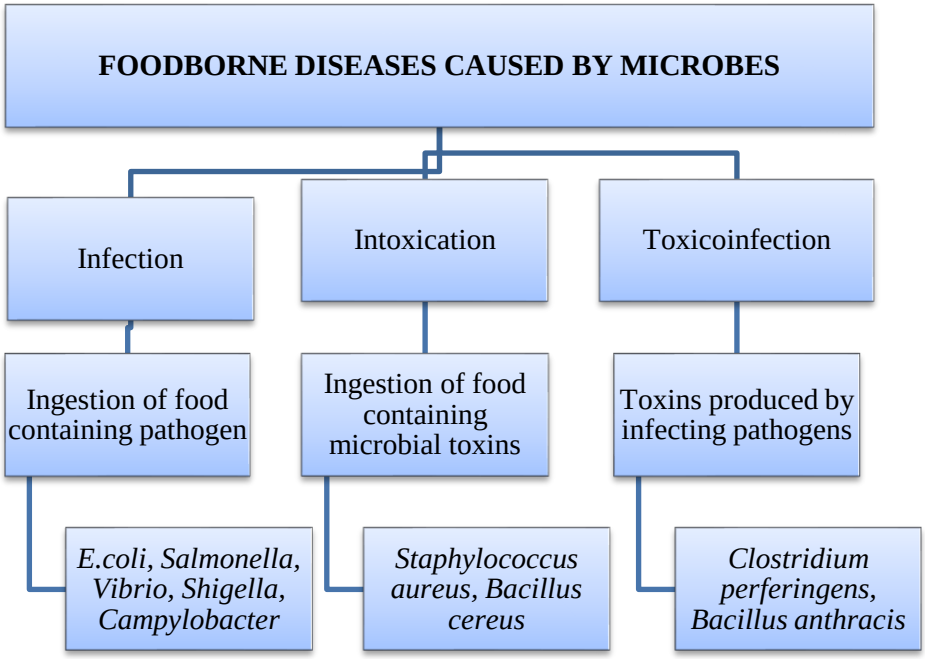

Figure 1- FOODBORNE DISEASES CAUSED BY MICROBES

1.3 Global scenario

Over 200 foodborne diseases have been identified to be caused by consumption of toxic food contaminated with bacteria, parasites, viruses, besides chemical residues (Lee & Yoon 2021).

Table 2- Foodborne disease incidence in different regions of the world

Region	Reports of Illness	Reports of Death
African Region	>91 Million	137000
Eastern Mediterranean	>100 Million	37000
European region	23 Million	5000
Southeast Asia	>150 Million	>175000
Western Pacific region	125 Million	50000

1.4 Food intoxication

Ingestion of food containing toxins of chemical nature, or produced by microorganisms or bio toxicants or by poisonous substances that were added as additives intentionally or unintentionally to the foods can cause food poisoning and is referred to as Food intoxication. Food intoxication unlike infections have a short incubation period. (Le Loir *et al.*, 2003). Food intoxication as per definition can be categorized as shown in the Figure 2.

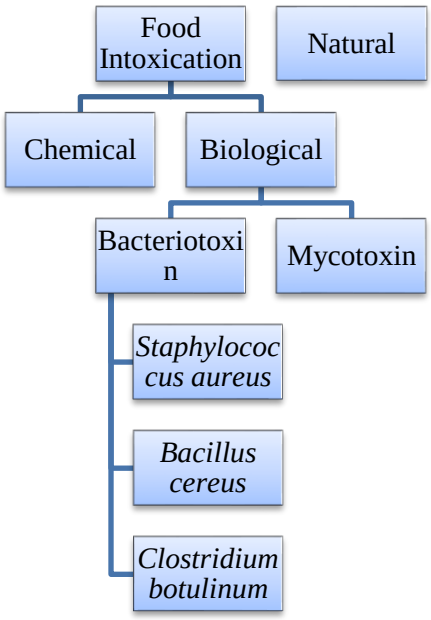

Figure 2- Types of food intoxication

1.5 Bacteriotoxins, their relevance and importance

The European Food Safety Authority and the European Centre for Disease Control (EFSA and ECDC, 2016), published data indicating that Foodborne diseases due to bacterial toxins was 19.5% in 2015 and was the third foodborne causative agent in the European Union.

1.5.1 Epidemiological significance of *Staphylococcus aureus* intoxication

Staphylococcal intoxication is one of the most widespread causes of foodborne intoxication globally.

Table 4-Selected major outbreaks associated with *S. aureus*

Year	No. of cases	Food	Country	References
Mid-1980s	>850	Chocolate milk	US	(Evenson et al. 1988)
1989	99	Canned mushrooms	US	(Levine et al. 1996)
1990	100	Ham	US	(Richards et al. 1993)
1998	4000 (16)	Chicken, roast beef, rice and beans	Brazil	(Do Carmo et al. 2004)
2000	13,420	Powdered skim milk	Japan	(Asao et al. 2003)
2006	113	Chicken and rice	Austria	(Schmid et al. 2007)
2007	400 (1)	UHT milk	Paraguay	(Weiler et al. 2011)

1.5.2 Epidemiological significance of *Bacillus cereus* intoxication

Table 5- Selected major outbreaks associated with *B.cereus*

Year	No. of outbreaks	Country	References
1998 – 2008	235	United States	Bennett *et al.*, 2005
2009 – 2015	65 (839 human cases)	United States	Dewey-Mattia *et al.*, 2018

2001 – 2013	6 (114 human cases)	Australia	May *et al.*, 2016
2007 – 2015	437	European Union	EFSA, 2016

1.6 Probiotics and their relevance

Probiotics are a type of friendly, beneficial species of microbes that have been extensively studied for their benefits in the recent past (Kumar *et al.*, 2016). Probiotics have multifold health benefits to the human body, some of which is summarized below in the Figure-3.

Figure 3-Health benefits of Probiotics

The term "Probiotics" was coined by German scientist Werner Kollath in 1953 (Gasbarrin *et al.*, 2016). The word probiotic comes from the Latin *pro* and the Greek *bios* which means 'for life' (Lilly *et al.*, 1965). In 2013, the Food and Agriculture Organization (FAO) defined probiotics as '*the live microorganisms administered in sufficient amounts that confer a health benefit on the host organism'* (FAO, 2002), in concurrence to the viewpoint of Elie Metchnikoff, made century ago (Mackowiak, 2013).

Many microorganisms are known to probiotic activities (Khaneghah & Fakhri, 2019), such as Lactic Acid Bacteria(LAB), *Bacillus* spp. (Shakibaie *et al.*, 2017), *Bifidiobacterium* etc.

2.0 REVIEW OF LITERATURE

2.1 *Staphylococcus aureus* as a Foodborne pathogen causing food poisoning or toxin mediated illness

2.1.1. Morphology and Cultural Characteristics

2.1.2.a. Morphology

Staphylococcus aureus are Gram-positive cocci that occur in groups as grape-like clusters.

2.1.2.b. Cultural Characteristics

S.aureus grows readily on ordinary media. They grow in a temperature range of 10°C to 42°C, the optimal being 37°C. A pH of 7.4 -7.6 is optimal for their growth, however, they can survive high salt concentrations

2.1.3 Relevance to human health

S.aureus are ubiquitous in nature and are also a part of normal flora of humans. They are primary parasites of human beings, colonizing the skin, skin glands and mucous membrane. They are the among the most common to cause infections in humans. They are known to cause common pyogenic infections of the skin and soft tissues, musculoskeletal tissues, respiratory tissues, central nervous system, endovascular system and urinary tract.

2.1.4 Occurrence and transmission

S.aureus have been found in a wide range of environmental sites such as soil, marine and fresh water, plant surfaces, dust and air .Transmission of the bacterium happens by direct or indirect contact with a person who is colonized with organism, either in wound discharges or clinical infections of respiratory or urinary tract.Raw meat is a good medium for S. aureus survival. Furthermore, food handlers carrying *S. aureus* on their gloves or bodies can also contaminate food (Wang *et al.,* 2017).

2.1.5 Pathogenicity & Clinical features with reference to food intoxication

Food poisoning by *S.aureus* is characterized by a short incubation period, typically 2-4 hrs. The ailment is self-limiting and the recovery is normally complete within 1-2 days. The symptoms include Nausea, vomiting, stomach cramps, retching and prostration that are mostly predominant. Diarrhoea may or may not occur.

2.1.6 Staphylococcal toxins and their types

The toxin mediated illness by *S.aureus* is caused through the secretion of enterotoxins produced by the bacterium. More than 20 types of enterotoxins are produced by *S.aureus* (SE toxins). Enterotoxins A (SEA), B (SEB), C_1 (SEC_1), C_2 (SEC_2), C_3 (SEC_3), D (SED), E (SEE), G (SEG), H (SEH), I (SEI), J

(SE*l*J) (Balaban & Rasooly, 2000), K (SE*l*K) (Orwin *et al*., 2001), L (SE*l*L), M (SE*l*M), N (SE*l*N), O (SE*l*O) (Jarraud *et al*., 2001), P (SE*l*P) (Omoe *et al*., 2005), Q (SE*l*Q) (Orwin *et al*., 2002), R (SER) (Omoe *et al*., 2003), S (SES), T (SET) (Ono *et al*., 2008), U (SE*l*U) (Letertre *et al*., 2003), and U2 and V (Thomas *et al*., 2006). These SE toxins belong to the pyrogenic exotoxins family. They have a low molecular weight of approx. 26,000 -30,000 (Bergdoll, *et al.*, 1989).

2.1.7 Staphylococcal Intoxication, cause and condition

When 20-100 ng of the toxin is ingested it can cause Staphyloccal Food Poisning (or intoxication). The intensity of illness depends on the dose pr amount of toxin, the age and health of humans (Asao *et. al.*, 2003). After the ingestion of toxin, the symptoms appear within a few hours. These include vomiting, diarrhea, nausea and abdominal pain (Bergdoll *et al.*, 1989). This disease is self-limiting and resolves within 24 to 48 hrs. So in most cases hospitalization is not a required but for infant, elderly and debilitated patients(Murray, R. J. 2005). *S. aureus* can reproduce in the range of temperature between 7℃ to 48.5℃, and a pH range between 4.2 to 9.3. In optimal conditions of temperature of around 35℃ and a pH around 7, *S. aureus* multiply rapidly. The SE toxins are produced and secreted when the numbers of cells are greater than 10^5 per gram of food.

2.2 *Bacillus cereus* as a Foodborne pathogen causing food poisoning or toxin mediated illness

2.1.2. a. Morphology

Bacillus cereus are Gram-positive rods that occur in chains.

2.1.2.b. Cultural Characteristics

Bacillus cereus grow readily on nutrient agar or peptone media. The bacterium is mesophilic and can adapt to a wide range of environmental conditions. The optimum temperature for growth is 37°C, but can grow in temperatures ranging from 20°C to 40°C. A pH range of 7.4 -7.6 is optimal for their growth. They are able to grow either in the presence or in the absence of oxygen.

2.2.3 Relevance to human health

B. cereus is most commonly associated with food poisoning. Gastroenteritis caused by *B. cereus* is mediated by the expression of enterotoxins. *B. cereus* is also known to cause opportunistic infections including posttraumatic eye infections, endocarditis, and bacteremia. Disease besides food poisoning is mediated through toxins namely, **necrotic toxin, cereolysin,** and **phospholipase C.**

2.2.4 Occurrence and transmission

Bacillus cereus is an aerobic spore-forming bacterium that is commonly found in soil, on vegetables, and in many raw and processed foods. It is commonly isolated from soil, vegetables, milk, cereals, spices, fried rice, cooked poultry and meats, soups, and desserts. It is also found in mashed potatoes, beef stew, apples, hot chocolates sold in vending machines, and other improper food handling areas. The bacterium is a saprophytic species of Bacillus. The bacterium interacts with other microorganisms in the rhizosphere, the region surrounding plant roots.

B.cereus is most commonly associated with food poisoning. *B. cereus* food poisoning may occur when foods are prepared and held without adequate refrigeration for several hours before serving, with *B. cereus* reaching $>10^6$ cells/g.

2.2.5.Pathogenicity & Clinical features with reference to food intoxication

Gastroenteritis caused by *B. cereus* is mediated by one of two **enterotoxins,** the heat-stable, proteolysis-resistant enterotoxin causes the emetic form of the disease and the heat-labile enterotoxin causes the diarrheal form of the disease. The emetic form is manifested by nausea, vomiting, abdominal cramps, and occasionally diarrhea and is self-limiting, with recovery occurring within 24 hours (Kramer & Gilbert, 1989). The diarrheal form has an incubation period of 1–24 hours and is manifested by profuse diarrhea with abdominal pain and cramps; fever and vomiting are uncommon (Granum, 1994).

2.2 Bacillus cereus toxins and their type

2.2.6.a Diarrheal toxins

There are five types of heat-labile diarrheal toxins which are produced by *B.cereus*, hemolysin BL and non-hemolytic enterotoxin, and the other three are FM, enterotoxin T and cytotoxin K. These are single protein enterotoxin protein substances (Beecher *et al.*, 1995, Lund *et al.*, 2000). Three-component HBL enterotoxin complex proteins L1, L2, L3 and b are encoded by genes hbl D, hbl C and hbl A respectively. Non-hemolytic enterotoxin (Nhe), is a three protein complex *Nhe* A, *Nhe* B and *Nhe* C(Krause *et al.,* 2010). The third enterotoxin is cytotoxin k (cyt K) also known as Ent K. It is encoded by cyt K gene (Krause *et al.,* 2010). Enterotoxin T is also a type of toxin (bceT) associated with lethal effect. (Agata *et al.,* 1995)

2.2.6.b Emetic toxins

The emetic toxin is identified as a ring-structure peptide composed of three repeated sequences of four amino acid/ oxy- acid , which term Cereulide (molecular weight 1.2 k Da) and the following composition [d-O-leu-d-ala-l-O-val-l-val]3. It is produced by nonribosomal peptide synthetase, which is encoded by the Cereulide synthetase cluster of genes (Ehling-Schulz, *et al.*, 2006).

2.2.7 *Bacillus cereus* Intoxication, cause and condition

The production of enterotoxin varies strain to strain of *B. cereus*. The total infecting dose seems to vary between about 10^5 to 10^8 viable cells. Thus food containing more than 10^3 B. *cereus* cannot be considered safe for consumption (Granum & Lindbäck 2012). In diarrheal illness, the organism produces the toxin responsible in the small intestine and the infective dose is 10^4-10^9 cells per gram of food. And in case of the emetic toxin which is preformed and ingested along with the food the concentration of the cells needs to be about 10^5-10^8 cells per gram in order to produce sufficient toxin. Enterotoxin is produced in the temperature range 10-43 °C with optimum temperature range of 32°C. Under reducing oxygen levels, more toxin is produced in the small intestine (Krause *et al.*, 2010). Emetic toxin is produced over a temperature range of 12–37°C but the optimum temperature is 12–15°C (Bauer *et al.*, 2010)

Diarrheal syndrome

B.cereus produces toxins during the exponential and stationary phase of growth in the small intestine (Stenfors Arnesen. *et al.*, 2008). Three enterotoxins are associated with diarrheal syndrome. Hemolysin BL (Hbl) has hemolytic ability and forms transmembrane pore for osmotic lysis, the nonhemolytic enterotoxin (Nhe) causes transmembrane pore and the cytotoxin k (cyt K) also known as EntK causes necrosis and hemolysis(Lund *et al.*, 2000).

Emetic syndrome

Cereulide emetic toxin is a mitochondrial toxin and can be absorbed from the gut to the bloodstream (Paananen. *et al.*, 2002), acting as a potassium ionophore resulting in dissipation of the transmembrane potential. It stimulates swelling and affects respiration resulting in mitochondria inactivation and also inhibits human natural killer cells. When Cereulide binds to the 5-ht3 receptor

and stimulates the vagal afferent nerve leading to stimulation of the vomiting center of the brain (Bauer *et.al.,* 2010).

2.3 Probiotic in disease prevention and therapy

2.3.1. Benefits of Probiotics for Health in General

The term 'Probiotics', of Greek origin etymologically means *'for life'*, which has a large implication in terms of the health benefits that could be derived from them. The reported benefits of probiotic consumption include

- ✓ Improvement of intestinal health
- ✓ Improvement of symptoms lactose intolerance
- ✓ Reduction of risk of various other diseases
- ✓ Enhancement of Immune response
- ✓ Reduction of serum cholesterol
- ✓ Cancer prevention

There have been a plethora of research reports that deliberate on the possible role of probiotics in health improvement and the Table-7.0 lists the same.

Table 7 Disease or Disorder

Disease or Disorder	Reference
Atopic diseases	Pierau *et al.,* 2021
Acute and antibiotic disorder	Vandenplas, 2020
Acute Upper Respiratory Infection	Emre *et al.,* 2020
Colic	Shirzinia *et al.,* 2021
Colon cancer	Yue *et al.,* 2020
Constipation	Dimidi *et al.,* 2020
Diabetes & Obesity	Wang *et al.,* 2020
Irritable Bowel Syndrome	Selvaraj *et al.,* 2020
Kidney Stores	Tavasali *et al.,* 2021
Modulation of Gut-Brain Axis	Morl *et al.,* 2020

Of the many strains of probiotics, *Lactobacillus* and *Bifidiobacteria* are largely in use. Table – 8.0 Lists some of the strains of the duo that are considered probiotic and under use.

Table 8 *Lactobacillus* Species

***Lactobacillus* Species**	***Bifidiobacterium* Species**
L.acidophilus	*B.longum*
L.casei	*B.bifidum*

L.plantarum	*B.lactis*
L.rhamnosus	*B.breve*

Among these, many probiotics species have been reported to be of use in therapy and treatment of infectious diseases. *Lactobacillus Spp* in the treatment of Urogenital infections (Bacterial vaginitis)(Neget, 2018),*Lactobacillus gasseri* and *L.fermentum* in the treatment of periodontal diseases (Jayaram *et al.,* 2016) are to name a few. On the other hand it also helps to maintain the balance of the gut microbiome and improve the response of the immune system by immunomodulation that generally act by –

- ✓ Enhancing the gut epithelial barrier (Madsenk, 2012)
- ✓ Altering the mucus secretion (Mays *et al.,* 2020)
- ✓ Increasing the activity of macrophages or natural killer cells, (Ivec *et al.*, 2007)
- ✓ Modulating the secretion of immunoglobulin or cytokines, (Fukushima *et al.,* 1998)
- ✓ Competitive exclusion of other (pathogenic) bacteria (La Fata *et al.*, 2018)

2.3.2. Probiotics for use against pathogens

Probiotics have been interesting research findings on the antimicrobial activities of probiotics against pathogens both on animal models and invitro studies. Table-9.0 & Table -10.0, detail the research findings of the anti-pathogenic activity of specific probiotics invitro and in animal models.

Table 9 *In vitro* studies on the antibacterial activity of probiotics on pathogens

Probiotic Strains	Pathogen species	Reference
Lactobacillus plantarum	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Candida albicans* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Thomas *et al.,* 2011 Lopez *et al.,* 2017
Lactobacillus fermentum	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Methicillin Resistant S. aureus* *Trichophyton mentagrophytes* *Trychophyton rubrum*	Jones *et al.,* 2010
Lactobacillus reuteri	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Candida albicans* *Enterobacter hormaechei* *Klebsiella pneumoniae* *Acinetobacter baumannii*	Chan *et al.,* 2018

Lactobacillus casei	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Candida albicans* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Li *et al.*, 2018 Lopez *et al.*, 2017
Lactobacillus rhamnosus	*Pseudomonas aeruginosa* *Staphylococcus aureus*	Al-Malkey *et al.*, 2017
Lactobacillus delbrueckii	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Lopez *et al.*, 2017
Lactobacillus acidophilus	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Al-Malkey *et al.*, 2017 Lopez *et al.*, 2017 Soleymanzaleh *et al.*, 2018
Lactobacillus brevis	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Lopez *et al.*, 2017
Lactobacillus salivaris	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Lopez *et al.*, 2017
Bifidobacterium bifidum	*Pseudomonas aeruginosa*	Soleymanzaleh *et al.*, 2018
Bifidobacterium longum	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Lopez *et al.*, 2017
Bifidobacterium lactis	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Lopez *et al.*, 2017
Bifidobacterium animalis	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Escherichia coli* *Propionibacterium acnes* *Propionibacterium aeruginosa*	Lopez *et al.*, 2017
Bacillus coagulans	*Pseudomonas aeruginosa*	Soleymanzaleh *et al.*, 2018
Propionibacterium acnes	*Methicillin Resistant S.aureus*	Shu *et al.*, 2013

Table 10 Animal model studies on the antibacterial activity of probiotics on pathogens

Probiotic Strains	Pathogen species	Reference
Lactobacillus plantarum	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Methicillin Resistant S.aureus*	Valdez *et al.*, 2005 Ong *et al.*, 2019 Surmeli *et al.*, 2019
Lactobacillus fermentum	*Staphylococcus aureus*	Jones *et al.*, 2012
Propionibacterium acnes	*Methicillin Resistant S.aureus*	Shu *et al.*, 2013

2.4 EFFECT OF PROBIOTICS ON MICROBIAL TOXIN PRODUCTION

2.4.1 Inhibitory effect of probiotics on Mycotoxins

Certain group of fungi belonging to the genera *Aspergillus, Penicillium, Fusarium* and *Alternaria* produce secondary metabolites which otherwise are termed as Mycotoxins, that are produced in foods at ambient conditions(Groopmanelal, 2012). Among the list of mycotoxins as shown in the Table-11.0, Aflatoxins (B1, B2, G1 and G2) are the most common (Muhialdin *et al.*, 2020).

Table 11- Mycotoxins

Fungi	Toxins produced
Aspergillus Spp	Aflatoxins Ochratoxins Patulin
Fusarium Spp	Dioxynivalenol Fumonisins T-2 toxin Zearalenone
Penicillium Sps	Patulin Ochratoxin

A variety of LAB strains have been screened for possible applications in mycotoxin degradation in foods and feeds, Table-12.0 lists the same.

Table 12 Mycotoxins degraded

LAB Strains	Mycotoxins degraded	Reference
Lactobacillus rhamnosus	Aflatoxin B1 Ochratoxin A Zearalenone	EL- Nezami *et al., 1998* Luz *et al.*, 2018 *Rogowska et al.*,2019
Lactobacillus casei	Aflatoxins (B1, B2, G1, G2)	Niderkorn *et al.*,2009
Lactobacillus plantarum	Aflatoxins (B1, B2, G1, G2, M1) Dioxynivalenol Zearalenone	Niderkorn *et al.*,2009 Franco *et al.*,2011 Cvek *et al.*,2012
Lactobacillus lactis	Aflatoxin B1	Sezer *et al., 2013*

	Zearalenone	Rogowska *et al.*,2019
Lactobacillus brevis	Aflatoxin B1 Ochratoxin A Patulin	Niderkorn *et al.*,2009 Wong *et al.*, 2015
Lactobacillus bulgaricus	Aflatoxin M1	Elsanhoty *et al.*,2014
Lactobacillus acidophilus	Ochratoxin A Patulin	Fuch *et al.*, 2008

Evidently, research prospects, screening for possible applications of probiotics in biological detoxification of mycotoxins has shown progress. However, LAB as probiotics with potentials as biological detoxifying agent needs to be explored.

2.4.2. Inhibitory effect of probiotics on Bacterial toxins

Foodborne disease caused by ingestion of bacterial toxin is termed *'Food Intoxication'* and as 'Food poisoning' in layman terminology. The most common of food intoxication as detailed in the sections earlier are caused by toxins produced by *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium botulinum.* The epidemiological significance and relevance of bacterial food intoxication has already been discussed in detail in the preceding section. Toxins released by the food intoxicating species of relevance are as shown below in Table-13.0

Table 13 Toxins produced

Bacteria	Toxins produced
Clostridium botulinum	Botulinum toxin Alpha toxin Beta toxin
Bacillus cereus	cytotoxin k & emetic toxin
Staphylococcus aureus	Enterotoxin (A, B, C,D, E, F, G, H, I, J)

2.5 ROLE AND RELEVANCE OF *Lactobacillus* Spp

Table 14- Significance of *Lactobacillus* Spp in context

Probiotics	Inhibitory Effect on Pathogens	References
L. plantarum	Isolates from fecal sample which serve as antimicrobial agents against *Salmonella*, *Shigella* and *E. coli* that contaminate food and cause human diseases.	Darbandi *et al.*, 2021
L.reuteri, *L.acidophilus* *L. plantarum*	These probiotics produce bacteriocin and organic acid which are antagonistic against *S.aureus, Salmonella, Listeria monocytogenes* and *C. perfringens*.	Ahmadnejad, E., & Dolatabadi, S. (2021)

L.reuteri KUB-ACS	High dose of *L. reuteri* given to chickens after the three days of hatching, it prevented Salmonella infection in chickens.	Nakphaichit *et al.*, 2019
L.casei	When the orally immunized with *L.casei*, mice induced humoral and cellular immunity inhibiting the effect of alpha toxin.	Gao, *et al.*, 2019.
Lactobacillus casei Shirota	In vivo experiment with *Lactobacillus casei* Shirota showed high capacity to bind with AFB1 and neutralize the toxicity of AFB1.	Liew *et al.*, 2018
Lactobacillus plantarum, Lactobacillus casei, Lactobacillus fermentum	*These isolates from dairy product have showed strong binding capacity with AFB1 and detoxified approximately 25%-61% at 37 ℃.*	Fazeli, *et al.*, 2009
Lactobacillus casei L. acidophilus	Both strains showed inhibitory effect on the Shiga toxin producing *E.coli* (STEC) O157:H7	Ogawa *et al.*,2001, Hernandez-Mendoza,*et al.*, 2010
Lactobacillis species	Homofermantative lactobacillis isolated from kefir grain have strong inhibitory activity against *Salmonella typhimurium* and *E.coli.*	Golowczyc, *et al.*, 2008
Lactobacillus plantarum	The subunit B of type-II Shiga toxin binds with cell wall and decrease the cytotoxicity.	Kakisu, *et al.*, 2013
L. plantarum L. paracasei L. fermentum	AFM 1 binds with cell wall of these probiotics after washing with phosphate buffer solution.	Cruz, *et al.*, 2021
Saccharomyces cerevisiae Lactobacillus rhamnosus, Lactobacillus delbrueckii Bifidobacterium	*These were* heat killed by ultra-high temperature. These heat killed organism have potential to reduce the concentration of AMF1 in milk.	Corassin, *et al.*, 2013

2.6 ROLE AND RELEVANCE OF *Bifidobacterium* Spp

Table 15- Significance of *Bifidobacterium* Spp in context

Probiotics	Inhibitory Effect on Pathogens	References
B. thermophilum B. boum B. suis B. animalis	These probiotics produce organic acid which decrease the pH value and influence Shiga toxin gene expression	Carey, *et al.*, 2008
Bifidobacterium bifidum Bifidobacterium catenulatum	Probiotics have anti infectious activity against *Escherichia coli* 0157H. the high concentration of acetic acid and low pH was found to inhibit Shiga toxin production	Asahara,*et.al.*, 2004
B. longum	The Probiotics in yoghurt, inhibits	Elkholy, *et al.*, 2014

	the growth of *S.*aureus during refrigeration.	
B. thermacidophilum *B. thermophilum*	*Listeria monocytogenes* is effectively inhibited by these probiotics (about 38% to 90%)	Moroni.,*et al.*,2006
Bifidobacterium	AFM1 binds the polysaccharide and peptidoglycan present in LAB cell wall and detoxified the mycotoxin.	Mahmood, *et al.*, 2018
Bifidobacterium	Heat killed Bifidobacteria have binding capacity with AFB1. The binding efficiency about 25%-60% .	Oatley, *et al.*,2000

3.0 RATIONAL TO STUDY

Food intoxication due to *S.aureus* & *B.cereus* have always been a topic of concern globally, and hence intriguing. Since the advent of antimicrobial usage, it has been the most sought for among the options available, to control microbes, in various habitats, food being one. Unlike the infectious, disease causing bacterial agents such as *E.coli* or *Salmonella*, *S. aureus* and *B. cereus* produce and secrete toxins in food, which when consumed causes food poisoning. The toxin producing pathogens, do so only in conditions of high population density. The object therefore is to contain the growth of pathogens in food. Needless to mention the use and abuse of antimicrobial for the said purpose, which has resulted in the emergence of multidrug resistant strains. Probiotics, given its diversified health benefits could be a suitable alternative to competitively exclude foodborne pathogens, including *S.aureus* and *B.cereus*. This therefore warrants a study to understand the possibility of putting probiotics to use for pathogen control and control of expression of toxins produced by them.

4.0 OBJECTIVES

- To study the effect of selected strains of *Lactobacillus (L.casie and L. plantarum)* on the growth and proliferation of a food intoxicating bacteria, namely *Staphylococcus aureus* and *Bacillus cereus*.
- To study the effect of *Bifidobacterium bifidum* on the growth and proliferation of food intoxicating bacteria , namely *Staphylococcus aureus* and *Bacillus cereus*.
- To study the molecular response of food intoxicating bacteria, namely *Staphylococcus aureus* and *Bacillus cereus* to the presence of *Lactobacillus sps.*

- To study the molecular response of food intoxicating bacteria, namely *Staphylococcus aureus* and *Bacillus cereus* to the presence of *Bifidobacterium bifidum* .

5.0 PLAN OF STUDY & METHODOLOGY

Table 14 Plan of Study & Methodology

Steps	Procedure	Methodology
1	Culture Procurement	Standard National & International Culture Repositories Agricultural Research Services (ARS) Northern Regional Research Laboratory (NRRL), United States Department of Agriculture (USDA), Peoria, USA. National Collection of Industrial Microbes (NCIM), Pune, INDIA.
2	Culture Preservation & Maintenance	Glycerol Stock (Duetz *et al*,. 2000) Slant cultures (Serial Subculturing)
3	Screening for antagonism by Probiotic species on *S.aureus*	Co-culture studies in broth Enumeration of pathogens by culture plating a. Serial dilution (Desrosiers *et al*., 2007) b. Spread plating (Valiammai *et al*.,2021)
4	Screening for antagonism by Probiotic species on *B.cereus*	
5	Screening for expression of exotoxins by *S.aureus* & *B.cereus* in pure culture and co-culture conditions	Enzyme Linked Immuno Sorbent Assay (ELISA) (Bennett, R. W. 2005) or High Performance Liquid Chromatography (HPLC) (Kim *et al*,. 2010)
6	Differential gene expression by *S.aureus* & *B.cereus* in pure culture and co-culture conditions	Quantitative Polymerase Chain Reaction (Chen *et al*,. 2016) / Microarrays (Tirumalai *et al*,. 2011)
7	Differential gene expression by probiotic strains in the presence of *S.aureus* & *B.cereus*	qPCR / Microarrays (Tirumalai *et al*,. 2011)

- The ELISAs is work on specific antibiotic binds with target antigen and were specific for the toxoids being detected. It has been developed for quantifying exotoxin and toxin concentration in samples.
- High-performance liquid chromatography (HPLC) is an significant methodical method usually used to separate and quantify components of samples. In this technique, a solution (first phase) is pumped through a column that contains a packing of small porous particles with a second phase bound to the surface.

- Quantitative PCR allows the sensitive, specific and reproducible quantitation of nucleic acids. The entire transcription of specie is studied by qPCR.. It quantifies the expression level of genes; it could be used for a set of gene.
- Microarrays are used to detect the expression of thousands of genes at the same time. DNA microarray is based on principle of hybridization between the nucleic acid strands.

Culture Procurement

- Cultures are procured from Standard National & International Culture Repositories. *Lactobacillus plantarum, Bifidobacterium bifidu*m and *Staphylococcus aureus* are procured by Agricultural Research Services (ARS) Northern Regional Research Laboratory (NRRL), United States Department of Agriculture (USDA), Peoria, USA. and
- *Lactobacillus casei* and *Bacillus cereus* are procured by National Collection of Industrial Microbes (NCIM), Pune, INDIA.

Culture Preservation & Maintenance

- Culture are Preserved & Maintained through Glycerol Stock (Duetz *et al*,. 2000)
- Slant cultures (Serial Subculturing).

Screening for antagonism behavior

- Screening for antagonism by LAB on *S.aureus* and *Bacillus cereus* by Co- culture studies in broth then enumeration of pathogens by serial dilution and spread plating. Culture will be incubated for 24 hrs,48 hrs. and 72 hrs. Cell form unit will be calculated.

To Screening the expression of exotoxin

- Screening for expression of exotoxins by *S.aureus* & *B.cereus* in pure culture and co-culture conditions by using Enzyme Linked Immuno Sorbent Assay (ELISA) (Bennett, R. W. 2005) or
- High Performance Liquid Chromatography (HPLC) (Kim *et al*,. 2010).
- Differential gene expression by *S.aureus* & *B.cereus* in pure culture and co-culture conditions by Quantitative Polymerase Chain Reaction (Chen *et al*,. 2016) /Microarrays (Tirumalai *et al*,. 2011).

To study the Gene expression

- Differential gene expression by *S.aureus* & *B.cereus* in pure culture and co-culture conditions and also differential gene expression by probiotic strains in the presence of *S.aureus* & *B.cereus* by using qPCR / Microarrays (Tirumalai *et al*,. 2011).

6.0 EXPECTED OUTCOME

- The study undertaken is likely to give results on
- Possible antagonistic behaviour by strains of *Lactobacillus* Spp and *Bifidobacterium* Spp against *S.aureus* and *B.cereus.*
- Possible inhibitory action of strains of *Lactobacillus* Spp and *Bifidobacterium* Spp against toxin production by *S.aureus* and *B.cereus.*
- Both of which when confirmed can find applications as biocontrol mechanisms in foods, thereby reducing the (ab)use of antimicrobials and contain the emergence of antimicrobial resistance.

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